## REMARKS

### I. Preliminary Remarks

Claims 1 and 18 are amended herein, claims 2-4, 6-17 and 19-34 are canceled and new claims 36-42 are added herein. Support for new claims 35-42 can be found in the application as originally filed. See, for example, at page 7, line 9; page 13, lines 20-29 and 21, lines 14-25.

The cancellation of claims 2-4, 6-17 and 19-34 is not made for reasons pertaining to patentability. Applicants reserve the right to pursue the subject matter of any claim (whether original, amended or canceled) in continuing applications.

### II. The objections should be withdrawn.

The Examiner objected to Figure 3 because it is allegedly illegible. In response, a replacement Figure 3 is submitted herewith.

The Examiner objected to the specification because the specification refers to Figures 10-11 on page 25, but such Figures were not included in the Drawings. In response, the specification has been amended to delete reference to Figures 10-11.

The Examiner also objected to claims 1, 5, 18, 29, and 32-33 for reciting the phrase "represented by." The objection is moot in view of either the cancellation of the objected claim or the deletion of the phrase from the rejected claim.

### III. The rejection under 35 U.S.C. § 112, second paragraph, is moot.

The examiner rejected claims 29, 32 and 33 under 35 U.S.C. §112, first paragraph, as allegedly being indefinite for recitation of the phrase "sequence variant thereof." The rejection is moot in view of the cancellation of these claims. Accordingly, the rejection should be withdrawn.

### IV. The rejection under 35 U.S.C. § 112, first paragraph (enablement), should be withdrawn.

The Examiner rejected claims 1, 5, 18, 29, 32 and 33 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The rejection is moot in view of the amendments made herein.

Specifically, claim 1 as amended positively recites an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 1. New claims 35-42 are directed to an isolated polypeptide comprising amino acids 1-483 of SEQ ID NO: 1. The specification discloses a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 and also a polypeptide comprising amino acids 1-483 of SEQ ID NO: 1 and teaches one of ordinary skill how to make such polypeptides. See, for example, at page 30, lines 22-30 of the application as filed. Uses for the claimed polypeptides are also disclosed in the application as filed and would be appreciated by one of ordinary skill in the art. One exemplary use for the polypeptide would be to generate antibodies against the polypeptide. Moreover, data demonstrating that the claimed polypeptides are involved in the control of p53-induced apoptosis has been published by the inventor and colleagues. See, Slee et al., Oncogene, 23:9007-9016, 2004 set forth in Appendix A. Accordingly, the rejection of claims 1 and 18 (claims 5, 29, 32 and 33 being canceled) under 35 U.S.C. § 112, first paragraph, should be withdrawn.

## V. Conclusion

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Dated: December 23, 2008

Respectfully submitted,

Electronic signature: /Jeanne M. Brashear/56,301
Jeanne M. Brashear
Registration No.: 56,301
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Agent for Applicants

# APPENDIX A

Oncogene (2004) 23, 9007–9016

www.nature.com/onc

ORIGINAL PAPERS

# The N-terminus of a novel isoform of human iASPP is required for its cytoplasmic localization

Elizabeth A Slee[1,3], Sébastien Gillotin[1,3], Daniele Bergamaschi[1,3], Christophe Royer[1,3], Susana Llanos[1], Safia Ali[1], Boquan Jin[2], Giuseppe Trigiante[1] and Xin Lu*[,1,3]

[1]Ludwig Institute for Cancer Research, Imperial College School of Medicine, Norfolk Place, London W2 1PG, UK; [2]Department of Immunology, Fourth Military Medical University, Xian 710032, Peoples' Republic of China

**ASPP1 and ASPP2 are both proteins that interact with p53 and enhance its ability to induce apoptosis by selectively elevating the expression of proapoptotic p53-responsive genes. iASPP(RAI) is a third member of the family that is the most conserved inhibitor of p53-mediated apoptosis. Here, we have described iASPP, a longer form of iASPP(RAI), which at 828 amino acids is more than twice the size of iASPP(RAI). Using two antibodies that recognize both iASPP and iASPP(RAI), we report that this longer form of iASPP is the predominant form of the molecule expressed in cells. Like iASPP(RAI), iASPP also binds to p53 and inhibits apoptosis induced by p53 overexpression. However, whereas iASPP(RAI) is predominantly nuclear, the N-terminus of iASPP is entirely cytoplasmic, and the longer iASPP is located in both the cytoplasm and the nucleus. The effect upon subcellular localization of the longer N-terminus of iASPP means that this new, longer form of the molecule may be subject to greater regulation and provides another layer in the control of p53-induced apoptosis.**

*Oncogene* (2004) **23**, 9007–9016. doi:10.1038/sj.onc.1208088
Published online 18 October 2004

**Keywords:** p53; iASPP; apoptosis

## Introduction

The balance between cell division and cell death is vital for the maintenance of homoeostasis in metazoan organisms, and the disruption of this balance contributes to many pathological conditions. p53 is a transcription factor that is activated following genotoxic stress, which regulates the expression of genes that are involved in either cell cycle arrest or death. As a result, p53 acts to prevent potentially dangerous mutations being passed on during cell division by either halting the cell cycle until the damage has been repaired, or by inducing apoptosis in order to eliminate the damaged cell (Vogelstein *et al*., 2000; Vousden and Lu, 2002; Slee *et al*., 2004).

The ASPP family is a newly discovered family comprising three proteins – ASPP1, ASPP2 and iASPP – that interact with and modulate the behaviour of p53 (Iwabuchi *et al*., 1994; Naumovski and Cleary, 1996; Samuels-Lev *et al*., 2001; Bergamaschi *et al*., 2003; Slee and Lu, 2003). The unifying structural characteristic of these three proteins is a homology at their C-termini, which contain a proline-rich region, four ankyrin repeats and an SH3 domain. It is through the ankyrin repeats and SH3 domain that these three proteins interact with p53 (Gorina and Pavletich, 1996). ASPP1 and ASPP2 enhance the ability of p53 to induce apoptosis by causing p53 to specifically upregulate the expression of proapoptotic genes rather than genes involved in cell cycle arrest. ASPP1 and ASPP2 also induce apoptosis via the p53 homologues p63 and p73 in a similar manner (Bergamaschi *et al*., 2004). The third family member, iASPP, was originally identified as a protein that interacts with and inhibits NFκB p65 RelA, and was named Rel-associated inhibitor (RAI) (Yang *et al*., 1999). iASPP/RAI is substantially shorter than ASPP1 and ASPP2, and inhibits p53-mediated cell death (Bergamaschi *et al*., 2003).

*ape-1*, a gene encoding an ASPP homologue, has been identified in *Caenorhabditis elegans* (Bergamaschi *et al*., 2003). Nematodes that have had *ape-1* eliminated by RNAi were observed to have an elevated number of germ cell corpses, which suggests that *ape-1* encodes a homologue of iASPP (Ce-iASPP) rather than ASPP1 or ASPP2. The observed number of corpses in *ape-1*$^{-/-}$ nematodes did not increase following DNA damage, and germ cells lacking both the p53 homologue *cep-1* and *ape-1* do not display the corpses seen in those nematodes lacking *ape-1* alone. This establishes that *cep-1* (*C. elegans* p53) and *ape-1* (Ce-iASPP) lie in the same pathway that leads to death following DNA damage, and that they act as mutual antagonists. Reflecting this, iASPP is also able to inhibit p53-mediated apoptosis in mammalian cells. However, Ce-iASPP is the only ASPP molecule present in *C. elegans* (Derry *et al*., 2001; Schumacher *et al*., 2001; Bergamaschi *et al*., 2003).

*Correspondence: X Lu; E-mail: x.lu@imperial.ac.uk
[3]Current address: Ludwig Institute for Cancer Research, University College London, 91 Riding House Street, London W1W 7BS, UK
Received 27 July 2004; revised 13 August 2004; accepted 16 August 2004; published online 18 October 2004

Our investigations have shown that the functions of Ce-iASPP and human iASPP/RAI are interchangeable, yet it is uncertain why Ce-iASPP is located in both the cytoplasm and the nucleus, whereas iASPP/RAI localizes exclusively to the nucleus. Secondly, it was unknown why the nematode protein is around twice the size of its human counterpart (Bergamaschi *et al*, 2003), which raises the possibility that either evolutionary selection has reduced iASPP in size, or there exists another, larger, mammalian iASPP protein. In this study, we have identified and characterized an 828 amino-acid iASPP protein that is expressed in mammalian cells. The longer isoform of iASPP is also an inhibitor of p53-mediated apoptosis, although like Ce-iASPP, the longer form of iASPP is localized to both the cytoplasm and the nucleus.

## Results

### *Identification of a longer, 828 amino-acid form of iASPP/RAI*

iASPP/RAI was originally isolated in a yeast two-hybrid assay using a fragment of NFκB p65 as a bait to screen human placenta and brain cDNA libraries. A partial cDNA isolated in the screen was used to isolate the full-length RAI cDNA from a human placenta cDNA library. This yielded two cDNAs, one of 2.1 kb (Accession number AF078036) and a second of 2.6 kb (AF078037), both containing the same open reading frame encoding RAI but differing in their 5′ untranslated regions (Yang *et al*., 1999).

A BLAST search of the National Centre for Biotechnology Information (NCBI) database using the iASPP/RAI cDNA sequence uncovered three ESTs that were highly homologous to iASPP/RAI but were over 3 kb in length. The open reading frame from one of these ESTs (Accession number BC032298) encoded a protein of 828 amino acids whose C-terminus was identical to that of iASPP/RAI (Figure 1a). A closer comparison between the DNA sequence of the EST and those isolated in the original study by Yang *et al.* revealed that the 2.1 kb cDNA was identical to but shorter than the BC032298 sequence. Exon–intron mapping shows that EST BC032298 and the 2.6 kb iASPP/RAI cDNA are derived from the same gene, with the sequence at the 5′ end of the 2.6 kb cDNA arising from another exon within the iASPP/RAI gene. The shorter ORF, which encodes RAI, arises as a result of a single base pair insertion that introduces a stop codon upstream of the RAI initiation codon. This frame shift was not present in any ESTs identified in the BLAST search, although it is present in both of the cDNA sequences in the original report. Furthermore, within the RAI open reading frame, a single base pair deletion has occurred followed, 84 base pairs later, by a single base pair insertion so that the reading frames of RAI and the EST sequence once more correspond. As a result, amino acids 562–590 of the new longer sequence are not found in the original RAI sequence (Figure 1a). Again, this frame shift within the RAI ORF was not present in any of the ESTs isolated in the BLAST search. None of these frame shifts are present in the genomic sequence. While ASPP1 and ASPP2 possess substantial sequence similarity within the N-terminal portion of their sequences, the new iASPP protein bears little homology to the other two family members aside from its C-terminus (Figure 1b and c). For clarity, the newly identified longer protein will hereafter be referred to as iASPP, whereas the shorter isoform will be referred to as iASPP(RAI).

### *The 828 amino-acid iASPP is the form predominantly expressed* in vivo

To provide evidence that iASPP exists *in vivo*, we obtained EST clone BC032298 and subcloned the cDNA into a mammalian expression vector in frame with a C-terminal V5 epitope tag. We also generated antibodies using different antigens contained within sequence common to iASPP and iASPP(RAI) (Figure 2a). The first antibody, a mouse monoclonal antibody named mAbiASPP49.3, was raised against a 180 amino-acid His-tagged fragment representing amino acids 479–659 of iASPP. Secondly, a peptide encoding iASPP amino acids 492–509 was used to generate mouse monoclonal antibody SA4.1 and rabbit polyclonal antibody pAbiASPP18. These antibodies were first tested against proteins generated from the iASPP and iASPP(RAI) cDNAs by *in vitro* translation. As shown in Figure 2b, the translation products of both cDNAs were detected by mAbiASPP49.3 and SA4.1. pAbiASPP18 was also able to detect both proteins (data not shown). Next, as mAbiASPP49.3 appeared to be the most effective antibody for blotting, we used this antibody to detect the presence of iASPP and iASPP(RAI) in a range of cell lines derived from different tissues. Extracts from normally growing RKO, Saos-2, Tera, H1299, 293, SK-MEL-37, MCF7 and U2OS cells were analysed by immunoblotting alongside *in vitro* translated iASPP and iASPP(RAI) (Figure 2c). mAbiASPP49.3 detected a number of bands in the cell lysates. Importantly, a band that ran at approximately 100 kDa was the same size as the *in vitro* translated product of the cDNA derived from EST clone BC032298. Although mAbiASPP49.3 was able to detect the *in vitro* translated iASPP(RAI), no bands of equivalent size could be seen in any of the cell lysates under these conditions.

To determine which of the bands detected in the cell lines shown in Figure 2c by antibody mAbiASPP49.3 correspond to the iASPP cDNA, MCF7 cells were transfected with the plasmid encoding iASPP and the resulting cell lysate analysed using mAbiASPP49.3 alongside cells transfected with empty vector (Figure 3a, left panel). A band of equivalent size to the 100 kDa band seen in the untransfected cells was intensified in the cells transfected with the iASPP plasmid, as was a band that migrated at approximately 70 kDa. Next, mAbiASPP49.3 was able to detect the 100 kDa and the 70 kDa band following immunoprecipitation with anti-V5 antibody using H1299 cells that stably express iASPP carrying an N-terminal V5 tag, so

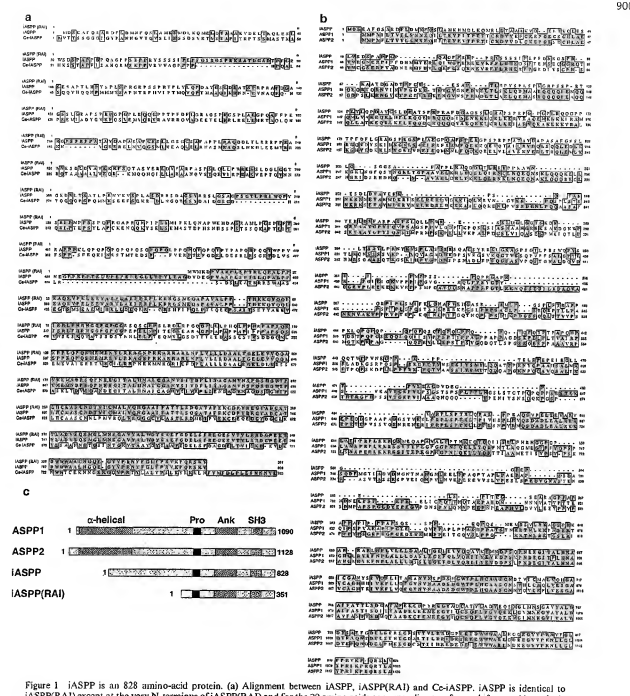


Figure 1 iASPP is an 828 amino-acid protein. (a) Alignment between iASPP, iASPP(RAI) and Ce-iASPP. iASPP is identical to iASPP(RAI) except at the very N-terminus of iASPP(RAI) and for the 29 amino acids corresponding to a frame shift caused by a single base pair deletion and subsequent insertion in the iASPP(RAI) cDNA. Although Ce-iASPP is of a similar length to iASPP, the homology between the two proteins aside from that seen at the C-terminus is low. (b) Alignment between iASPP, ASPP1 and ASPP2. iASPP has significant homology with ASPP1 and ASPP2 at the C-terminus, which contains the ankyrin repeats and SH3 domain. ASPP1 and ASPP2 share considerable homology throughout the rest of their sequences, especially within their N-termini that are thought to be α-helical. iASPP does not share this homology. The ClustalW alignments in both (a) and (b) were generated using MacVector software. Dark shading indicates amino-acid identity except where only one sequence is present. (c) Diagram illustrating the relative lengths of ASPP1, ASPP2, iASPP and iASPP(RAI) and their regions of shared homology

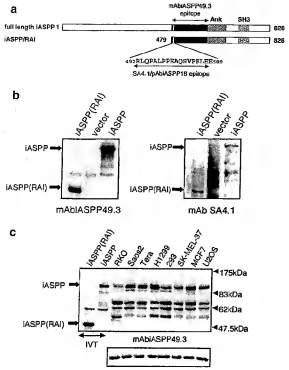


**Figure 2** iASPP can be detected by distinct antibodies raised against sequences within both iASPP and iASPP(RAI). (a) Diagram illustrating the relative positions of the antigens used to generate antibodies mAbiASPP49.3, SA4.1 and pAbiASPP18. (b) Both iASPP and iASPP(RAI) cDNAs were translated *in vitro* using unlabelled amino acids. A control reaction was run alongside which contained empty vector. The band observed between the two iASPP proteins in the blot probed with mAbiASPP49.3 is nonspecific. (c) Expression levels of iASPP and iASPP(RAI) were detected in cell lines using mAbiASPP49.3. *In vitro* translated products of the iASPP(RAI) and iASPP cDNAs (IVT) are loaded as positive controls. The positions of the molecular weight markers are shown on the right. Anti-PCNA antibody PC-10 was used as a loading control for the cell lysates

the bands being recognized by mAbiASPP49.3 must correspond to our cDNA and contain a V5 tag at the N-terminus. To determine whether the antibodies could immunoprecipitate endogenous iASPP, we used lysate prepared from both H1299 and U2OS cells (Figure 3b). When we used pAbiASPP18 to immunoprecipitate followed by mAbiASPP49.3 to detect the precipitated complexes on an immunoblot, two bands of 100 and 70 kDa could be seen. When the antibodies were reversed, that is, pAbiASPP18 was used to detect the proteins pulled down by mAbiASPP49.3, the same two bands were detected again.

Next, we transfected either a plasmid containing an iASPP siRNA or the parental vector into H1299 cells alongside pMACS H-2K$^{K}$, a plasmid encoding a cell surface selection marker that enables transfected cells to be separated from nontransfected cells. When the transfected and nontransfected cell populations were analysed for iASPP expression, cells carrying the iASPP siRNA showed a markedly reduced expression of the 100 and 70 kDa bands, whercas the other bands remained unaffected (Figure 3c). While both our antibodies can detect iASPP(RAI) when it is translated *in vitro*, a product of the equivalent size to iASPP(RAI) is not detected in any of the cell types used by any of the methods we employed, although another form of iASPP migrates at around 70 kDa that is likely to be a degradation product of the 100 kDa iASPP. Taken together, our data from Figure 3 demonstrate that the predominant form of iASPP in the cell types examined is a protein that migrates at around 100 kDa, and this corresponds to the EST clone isolated following the BLAST search.

### *iASPP interacts with p53* in vivo *and* in vitro

We have previously shown that iASPP(RAI) is an inhibitor of p53, so it was necessary to demonstrate that iASPP behaves in the same way. Firstly, we needed to determine whether the presence of the extended N-terminus of iASPP interferes with its ability to bind to p53. Anti-V5 antibody was used to immunoprecipitate *in vitro* translated iASPP and iASPP(RAI), both carrying a C-terminal V5 tag, in the presence and absence of *in vitro* translated p53, which is untagged (Figure 4a, left panel). p53 can be immunoprecipitated by anti-V5 antibody when either iASPP(RAI) or iASPP are present, hence iASPP is able to interact with p53 *in vitro*, and the N-terminus does not hinder this interaction. To confirm that the same is true *in vivo*, we used pAbiASPP18 to immunoprecipitate endogenous p53 and iASPP from MCF7 cells (Figure 4a, right panel). p53 is precipitated by pAbiASPP18, which also pulls down iASPP, thereby demonstrating that iASPP can also interact with p53 in the cell.

### *iASPP is an inhibitor of p53-mediated apoptosis*

The coexpression of ASPP1 or ASPP2 with p53 enhances the ability of p53 to induce apoptosis, and this is inhibited by iASPP(RAI) or Ce-iASPP. To ascertain whether iASPP behaves in a similar manner to iASPP(RAI), p53-null Saos-2 cells were transfected with either p53 or p53 and ASPP2 (Figure 4b). The expression of p53 in these cells induces approximately 20% apoptosis, and consistent with our previous observations this figure is substantially increased when p53 is expressed in combination with ASPP2. iASPP(RAI) and Ce-iASPP suppress the apoptosis induced by both p53 alone and by p53 and ASPP2 together, and iASPP also inhibits apoptosis under these circumstances to an equivalent degree. Therefore, the additional sequence at the N-terminus of iASPP does not prevent its ability to curb p53-mediated apoptosis, and the observation that Ce-iASPP, iASPP(RAI) and iASPP are all inhibitory demonstrates that it is the C-terminus of iASPP that is responsible for its antagonism of p53, as this region is common to all three molecules.

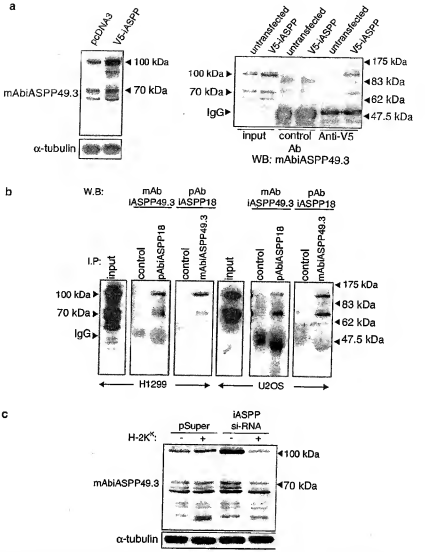


**Figure 3** iASPP is expressed *in vivo* as a protein of around 100 kDa. (a) MCF7 cells were transfected with either empty vector or a plasmid containing full-length iASPP cDNA, and then iASPP levels were determined by Western blotting using mAbiASPP49.3 (left-hand panel). H1299 cells stably expressing iASPP with an N-terminal V5 epitope were immunoprecipitated with anti-V5 antibody and the resulting immunocomplexes detected using mAbiASPP49.3 (right panel). (b) Endogenous iASPP was immunoprecipitated with pAbiASPP18 (left) or mAbiASPP49.3 (right) from both H1299 and U2OS cells, and detected by immunoblot with mAbiASPP49.3 or pAbiASPP18. The inputs were detected using mAbiASPP49.3. (c) H1299 cells were transfected with either pSuper, or pSuper containing iASPP siRNA, and pMACS H-2K$^K$ as a cell surface transfection marker that was used to separate the transfected (H-2K$^K$ +) from the untransfected (H-2K$^K$ −) cells. Levels of iASPP were detected using mAbiASPP49.3, and tubulin was used to indicate equal loading

### *N-terminus of iASPP contributes to its cytoplasmic location*

It has been shown that iASPP(RAI) localizes to the nucleus, whereas Ce-iASPP is also expressed in the cytoplasm (Bergamaschi *et al*., 2003). mAbiASPP49.3 was used to examine the localization of transfected and endogenous iASPP in Saos-2 and H1299 cells as indicated (Figure 5a). Both exogenous and endogenous iASPP have similar expression patterns. iASPP is predominantly expressed in the cytoplasm but small amounts of iASPP can also be detected in the nucleus, so its expression pattern is analogous to that of Ce-iASPP. When overexpressed, the amount of nuclear iASPP is increased (Figure 5b). To determine the region(s) of iASPP that affect localization, we made a

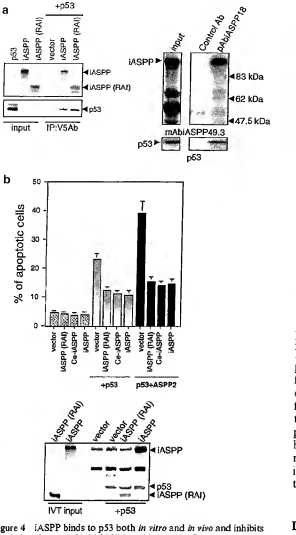


**Figure 4** iASPP binds to p53 both *in vitro* and *in vivo* and inhibits p53-induced apoptosis. (a) iASPP and iASPP(RAI) were translated *in vitro* in the presence of $^{35}$S-methionine, and p53 translated with unlabelled methionine. Both iASPP and iASPP(RAI) have C-terminal V5 tags whereas p53 has no tag. The translation products were combined as shown. Translated proteins were immunoprecipitated using an anti-V5 antibody, and iASPP and iASPP(RAI) were visualized by autoradiography. The presence of p53 was detected by Western blotting with anti-p53 antibody DO-1 (left panel). pAbiASPP18 was used to immunoprecipitate endogenous iASPP from MCF7 cells alongside a control antibody (right-hand panel). The presence of p53 was detected using antibody DO-1 and the blot was then reprobed using mAbiASPP49.3. (b) Saos-2 cells were transfected with plasmids encoding iASPP, iASPP(RAI) or Cε-iASPP either alone or in conjunction with p53 or p53 and ASPP2, using CD20 as a transfection marker. Apoptosis levels in the CD20-positive cells were then assessed by FACS. The Western blot illustrates the expression of the transfected proteins in the cells, alongside *in vitro* translated iASPP(RAI) and iASPP to act as a comparison. iASPP was detected using mAbiASPP49.3, and p53 was detected using anti-p53 antibody DO-1

series of iASPP deletions, all with a V5 epitope at their C-termini, and analysed their subcellular localization following transfection into Saos-2 cells (Figure 5b). Fragments A, B and D all localize primarily to the nucleus. All three of these fragments contain sequence that is present in the sequence shared by both iASPP and iASPP(RAI), which indicates that the presence of iASPP in the cytoplasm is due to its N-terminus. This is confirmed by the expression pattern of fragment H, which contains the sequence that is found in iASPP but not in iASPP(RAI), as this localizes exclusively to the cytoplasm. This suggests that the N-terminus acts in opposition to the C-terminus to enable iASPP to be present in the nucleus.

*Binding is required for iASPP to inhibit p53*

To assess what impact the N-terminus has upon the inhibitory action of iASPP, some of the truncations used in Figure 5 were transfected into Saos-2 cells with or without p53 and the levels of apoptosis measured by FACS. As shown in Figure 6a, none of the truncations induce apoptosis on their own. In conjunction with p53, however, only iASPP and iASPP(RAI) were able to inhibit apoptosis induced by p53 expression. Fragments A and B are unable to suppress apoptosis despite their nuclear location. Fragment H, which consists of the N-terminus of iASPP, is also unable to block p53-induced death. We also assessed the impact of fragment H upon the ability of p53 to elevate expression of the proapoptotic gene PIG3 by cotransfecting the fragment alongside a luciferase reporter plasmid containing the PIG promoter (Figure 6b). iASPP reduces p53-induced luciferase expression, whereas fragment H has no effect. Hence, the C-terminus of iASPP is the region of the molecule that is responsible for the inhibition of p53, and it is necessary for p53 and iASPP to directly interact for this inhibition to occur.

## Discussion

In previous studies, we have described the properties of ASPP, a new protein family that consists of ASPP1 and ASPP2, two proteins that both enhance the ability of p53 to induce cell death by elevating the expression of proapoptotic p53-responsive genes, and iASPP(RAI), a shorter homologue that suppresses p53-mediated cell death (Yang *et al*., 1999; Samuels-Lev *et al*., 2001; Bergamaschi *et al*., 2003). In this paper, we have described the properties of iASPP, an 828 amino-acid protein that is identical at its C-terminus to iASPP(RAI), but contains an additional 477 amino acids at its N-terminus.

The original paper describing iASPP(RAI) described two cDNAs, one of 2.1 kb and another of 2.6 kb that differs from the 2.1 kb cDNA in its 5′ untranslated region (Yang *et al*., 1999). Based on our study, the previously isolated 2.1 kb cDNA of iASPP(RAI) is a truncated form of iASPP. The 2.6 kb cDNA of iASPP(RAI), however, contains sequence from

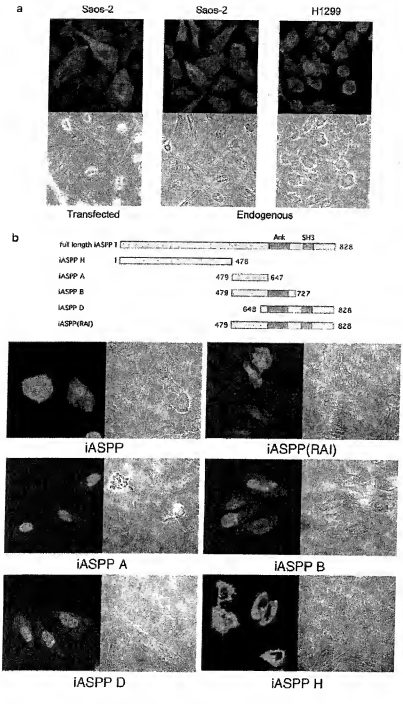


Figure 5 N-terminus of iASPP causes its localization to the cytoplasm. (a) mAbiASPP49.3 was used to detect iASPP in Saos-2 and H1299 cells. Either transfected (left) or endogenous iASPP (centre) was analysed in Saos-2 cells, alongside endogenous iASPP in H1299 cells (right). (b) V5 epitope-tagged constructs encoding the regions of iASPP illustrated in the diagram were transfected into Saos-2 cells and their subcellular localization determined by immunofluorescence using anti-V5 antibody. The accompanying panels show the corresponding phase-contrast images of the cells

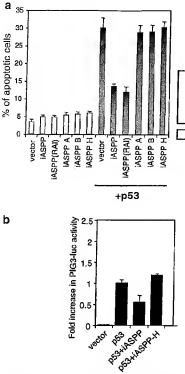


**Figure 6** C-terminus of iASPP is required for the inhibition of p53. (**a**) Saos-2 cells were transfected with p53 and the indicated iASPP truncations, and apoptosis was detected by FACS as for Figure 4b. The right-hand panel is an immunoblot showing the relative expression levels of the transfected proteins. (**b**) Saos-2 cells were transfected with 1 $\mu$g of a luciferase reporter plasmid containing the PIG-3 promoter alongside 50 ng of p53 and 0.25 $\mu$g of the iASPP plasmids. The graph shows the change in relative transactivation activity

another exon within the iASPP gene, so this could potentially be a splice variant. However, we have been unable to find any other complete cDNAs that contain this sequence in the EST database. If the single base pair insertion that causes the iASPP(RAI) reading frame is removed from this sequence, then in theory the new open reading frame would encode a protein of 403 amino acids. The persistent presence of the 100 kDa band in our blots and in our immunoprecipitations in addition to its correlation with the iASPP cDNA derived from the EST suggests that this is the predominantly expressed form of iASPP. Our inability to detect iASPP(RAI) could be indicative of a restricted tissue distribution, as iASPP(RAI) was isolated from a placenta cDNA library, and none of the cells used in this study are placental in origin. The 70 kDa protein is almost certainly a smaller fragment of iASPP as it is detected in cells transfected with iASPP carrying an N-terminal V5 tag by an anti-V5 antibody. The presence of the 70 kDa band in the immunoprecipitation shown in Figure 3a indicates that this is in all likelihood a degradation product representing the N-terminus of iASPP, as it must be derived from the cDNA which contains a V5 epitope at its N-terminus. The other bands detected are unlikely to represent iASPP(RAI) as the iASPP siRNA, derived from a region common to both iASPP and iASPP(RAI) failed to alter the expression levels of these bands. This siRNA does not affect the expre sion of ASPP1 or ASPP2, and there are no other ASPP family members in the human genome. Further investigation is required to determine whether they represent splice variants of iASPP or not.

In agreement with our previous observation that iASPP(RAI) is an inhibitor of p53, iASPP can also interact with and inhibit apoptosis induced by p53, demonstrating that the N-terminus of iASPP does not inhibit the activity of iASPP. While iASPP(RAI) is present almost exclusively in the nucleus, the presence of the extra amino acids at the N-terminus of iASPP(RAI) cause the partial relocation of iASPP from the nucleus to the cytoplasm and the effect of this portion of the protein can be clearly seen when the N-terminus alone (fragment H) is transfected and is expressed entirely in the cytoplasm. No classical nuclear localization signal is present in the C-termini of any of the ASPP proteins, although there is a precedence for ankyrin repeats to be able to promote nuclear import (Sachdev *et al*., 1998). This may not be the only mechanism for the nuclear localization of iASPP as fragment A, which does not incorporate the ankyrin repeats, is still present in the nucleus. Alternatively, it may be that the default location for iASPP is in the nucleus unless regulatory elements within the N-terminus actively transport the protein into the cytoplasm.

As iASPP(RAI) is almost entirely in the nucleus, the site where p53 and ASPP2 are exerting their effects upon proapoptotic gene expression, then the proportion of the protein able to act as an inhibitor of p53 is greater than that of iASPP. However, the presence of iASPP in the cytoplasm coincides with the distribution of ASPP1 and ASPP2, which are also predominantly cytoplasmic, so cytoplasmic iASPP may prevent ASPP1 and ASPP2 from binding to p53 in the cytoplasm. As p53 has been found in the mitochondria and the cytoplasm, regulation of p53 by the ASPP family may also be extranuclear and independent of transactivation (Mihara *et al*., 2003). The existence of protein sequence that may modulate the inhibitory effect of iASPP on p53 is important for a molecule that could influence whether or not a cell lives or dies. An iASPP(RAI) like molecule may be more oncogenic and hence could be selected for in tumour development. Nevertheless, its absence from any of the cell lines used in this study, all of which are tumour derived, indicates that this may not be a general phenomenon. More studies are needed to elucidate how the oncogenic function of iASPP is regulated during tumour development.

## Materials and methods

### *Cell culture and reagents*

Cells were grown in culture in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% foetal calf serum. The cells used in this study were Tera (testicular tumour cell line), RKO (colon carcinoma), Saos-2 (osteosarcoma), H1299 (lung carcinoma), 293 (embryonic kidney), SK-MEL-37 (melanoma), MCF7 (mammary epithelial) and U2OS (osteosarcoma). Anti-V5 antibody was purchased from Invitrogen. N-20 CD20Lu FITC-conjugated monoclonal antibody was from Becton Dickinson. Transfections throughout were performed by calcium phosphate precipitation.

### *Plasmids*

The EST containing the cDNA encoding iASPP (IMAGE clone 4994121) was obtained from MRC Geneservice (Cambridge, UK). The cDNA was subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen). pcDNA3.1 iASPP(RAI), pcDNA3.1 ASPP2, pcDNA3.1 Cε-iASPP and pcDNA3 p53 have been described previously (Samuels-Lev *et al*., 2001; Bergamaschi *et al*., 2003). The iASPP truncations used in Figure 5 were generated by PCR-directed cloning into pcDNA3.1/V5-His-TOPO. A modified pcDNA3 vector that has had two V5 sequences inserted 5′ of the polylinker was used to generate N-terminally V5-tagged iASPP.

### *Generation of anti-iASPP antibodies*

Anti-iASPP antibodies pAbiASPP18 (rabbit polyclonal) and SA4.1 (mouse monoclonal) were raised against the peptide RLQPALPPEAQSVPELEE (amino acids 492–509 of iASPP). Anti-iASPP mouse monoclonal antibody mAbiASPP49.3 was raised against a C-terminal His-tagged fusion protein containing amino acids 459–639 of iASPP. The corresponding cDNA was amplified by PCR and subcloned into pCRT7/CT-TOPO (Invitrogen). The recombinant iASPP fragment was generated in BL21 Star *E. coli* (Invitrogen) by incubation with 1 mM IPTG for 4 h followed by purification under denaturing conditions.

### *Electrophoresis and immunoblotting*

Cells were washed twice in PBS, then scraped into 1 ml PBS and pelleted at 400 *g*. The cells were lysed by incubating for 30 min at room temperature in 8 M urea, 1 M thiourea, 0.5% CHAPS, 50 mM DTT and 24 mM spermine, followed by centrifugation at 20 000 *g* for 20 min at 16°C. 30 *μ*g protein was used for analysis by SDS–PAGE and immunoblotting as described previously (Yap *et al*., 2000).

### *Immunoprecipitation*

Cells were lysed by incubating on ice in NP40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40 and protease inhibitors (Complete protease inhibitor cocktail, Roche)) for 45 min followed by centrifugation for 20 min at 20 000 *g* at 4°C. Between 0.5 and 2 mg lysate was precleared by rotating for 1 h at 4°C with protein G sepharose beads (Amersham Biosciences). Following removal of the beads, the lysate was transferred to a fresh tube and rotated overnight with blocked protein G sepharose beads at 4°C and approximately 1 *μ*g of either a specific antibody or nonspecific mouse or rabbit IgG (Sigma) as controls. The beads were then washed three times in ice-cold NP40 lysis buffer and the resulting complexes analysed by SDS–PAGE and immunoblot.

### *Construction and transfection of iASPP siRNA*

Oligonucleotides containing 19 bases of sequence present in both iASPP and iASPP(RAI) cDNAs were ligated into the pSuper expression plasmid as described previously (Brummelkamp *et al*., 2002). The plasmids were verified by sequencing. The complete sequences of the oligonucleotides used to generate the siRNA are as follows with the cDNA sequences shown in upper case: sense, 5′gatccccTGTCAAC TCCCCCGACAGCttcaagagaGCTGTCGGGGGAGTTGAC Attttggaaa 3′; antisense, 5′agctttccaaaaaTGTCAACTCCCC CGACAGCtctcttgaaGCTGTCGGGGGAGTTGACAggg 3′.

For transfection, $1 \times 10^6$ H1299 cells were plated into 10 cm dishes. Cells were transfected with 3 *μ*g of pMACS H-2K$^k$ alongside either pSuper or pSuper-si-RNA iASPP (10 *μ*g). At 48 h after transfection, cells expressing the pMACS H-2K$^k$ plasmid were separated using the MACS system (Miltenyi Biotec) according to the manufacturer's instructions. This gave rise to two populations of cells: H-2K$^k$ expressing (transfected) cells and nonexpressing (nontransfected cells). Both cell populations were lysed with RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 8, 1% NP40, 0.1% SDS) on ice for 30 min followed by centrifugation at 20 000 *g* for 30 min at 4°C.

### In vitro *translation and* in vitro *immunoprecipitation*

p53 and iASPP were translated *in vitro* with $^{35}$S-methionine using the TNT T7 Quick coupled Transcription/Translation System (Promega). The reticulocyte lysates containing each protein were combined as indicated and incubated together for 1 h at 30°C. mAbiASPP49.3 antibody immobilized on protein G sepharose beads was added to the binding reactions and rotated at 4°C for 16 h. The beads were then washed with PBS. The bound proteins were released in SDS sample buffer and analysed by 10% SDS–PAGE. Results were visualized by autoradiography.

### *Transactivation*

The transcriptional assay was carried out as described previously (Samuels-Lev *et al*., 2001).

### *Flow cytometry*

Saos-2 cells ($1 \times 10^6$) were plated in 10 cm dishes 24–48 h prior to transfection. All cells were transfected with 2 *μ*g of pCMV CD20 as a transfection marker. The following plasmids were transfected as appropriate at the stated amounts: pcDNA3 p53 (1 *μ*g), pcDNA3.1 Cε-iASPP (7.5 *μ*g), pcDNA3.1 iASPP(RAI) (7.5 *μ*g), pcDNA3.1 iASPP(1 *μ*g), pcDNA3.1 ASPP2 (10 *μ*g). A measure of 2 *μ*g iASPP truncations were used in Figure 6. Empty pcDNA3 vector was used to equalize the total amount of DNA in all samples. At 36 h after transfection, both attached and floating cells were harvested and analysed as described previously (Hsieh *et al*., 1997).

### *Immunofluorescence*

Saos-2 cells were seeded on coverslips in 24-well plates at 50% density and transfected with 0.5–3 *μ*g of plasmid encoding the iASPP truncations. At 24 h after transfection, the cells were fixed with 200 *μ*l of 4% paraformaldehyde in PBS for 12 min then permeabilized with 0.1% Triton X-100 in PBS for 4 min. Expression of the iASPP constructs

was detected using anti-V5 antibody (1:100 dilution in 0.2% fish skin gelatin) for 40 min followed by a TRITC or FITC-conjugated secondary antibody for 20 min.

## References

Bergamaschi D, Samuels Y, Jin B, Duraisingham S, Crook T and Lu X. (2004). *Mol. Cell. Biol.*, **24**, 1341–1350.

Bergamaschi D, Samuels Y, O'Neil NJ, Trigiante G, Crook T, Hsieh J-K, O'Connor DJ, Zhong S, Campargue I, Tomlinson ML, Kuwabara PE and Lu X. (2003). *Nat. Genet.*, **33**, 162–167.

Brummelkamp TR, Bernards R and Agami R. (2002). *Science*, **296**, 550–553.

Derry WB, Putzke AP and Rothman JH. (2001). *Science*, **294**, 591–595.

Gorina S and Pavletich NP. (1996). *Science*, **274**, 1001–1005.

Hsieh J-K, Fredersdorf S, Kouzarides T, Martin K and Lu X. (1997). *Genes Dev.*, **11**, 1840–1852.

Iwabuchi K, Bartel PL, Li B, Marraccino R and Fields S. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 6098–6102.

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM. (2003). *Mol. Cell*, **11**, 577–590.

Naumovski L and Cleary ML. (1996). *Mol. Cell. Biol.*, **16**, 3884–3892.

Sachdev S, Hoffmann A and Hannink M. (1998). *Mol. Cell. Biol.*, **18**, 2524–2534.

Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, Campargue I, Naumovski L, Crook T and Lu X. (2001). *Mol. Cell*, **8**, 781–794.

Schumacher B, Hofmann K, Boulton S and Gartner A. (2001). *Curr. Biol.*, **11**, 1722–1727.

Slee EA and Lu X. (2003). *Tox. Lett.*, **139**, 81–87.

Slee EA, O'Connor DJ and Lu X. (2004). *Oncogene*, **23**, 2809–2818.

Vogelstein B, Lane D and Levine AJ. (2000). *Nature*, **408**, 307–310.

Vousden KH and Lu X. (2002). *Nat. Rev. Cancer*, **2**, 594–604.

Yang J-P, Hori M, Sanda T and Okamoto T. (1999). *J. Biol. Chem.*, **274**, 15662–15670.

Yap DB, Hsieh JK and Lu X. (2000). *J. Biol. Chem.*, **275**, 37296–37302.

**Acknowledgements**

This work was funded by the Ludwig Institute for Cancer Research. DB was supported by a grant from the Association of International Cancer Research.